View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Kidney International, Vol. 60 (2001), pp. 786–796

Endothelial cell protection against ischemia/reperfusion injury by lecithinized superoxide dismutase

Dicken D.H. Koo, Kenneth I. Welsh, Nick E.J. West, Keith M. Channon, Anthony J. Penington, Justin A. Roake, Peter J. Morris, and Susan V. Fuggle

Nuffield Department of Surgery and Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom; Bernard O'Brien Institute of Microsurgery, St. Vincent's Hospital, Fitzroy, Victoria, Australia; and University Department of Surgery, Christchurch School of Medicine, Christchurch Hospital, Christchurch, New Zealand

Endothelial cell protection against ischemia/reperfusion injury by lecithinized superoxide dismutase.

Background. Organs used for transplantation may experience long periods of cold ischemic preservation and consequently oxygen free radical-mediated damage following reperfusion. Lecithinized superoxide dismutase (lec-SOD) is a novel free radical scavenger that has been shown to bind with high affinity to cell membranes. The aim of this study was to determine whether lec-SOD bound to endothelial cells under organ preservation conditions to mediate direct antioxidant activity at the endothelial cell surface and thus offer protection against the harmful effects of ischemia/reperfusion injury.

Methods. An in vitro study was performed on large vessel endothelial cells (HUVEC) and a human microvascular endothelial cell line HMEC-1, to investigate the potential therapeutic benefits of incorporating lec-SOD into organ preservation solution. A cold hypoxia/reoxygenation system was developed to examine lec-SOD binding affinity to endothelial cells, protection against hypoxia/reoxygenation-induced cell death, and neutrophil adhesion.

Results. Lec-SOD bound to endothelial cells with higher affinity than unmodified recombinant human superoxide dismutase (rhSOD) and significantly protected both HUVEC and HMEC-1 from cell death following 27 hours of cold hypoxia ($P < 0.01$). Furthermore, neutrophil adhesion to the endothelium stimulated by hypoxia and reoxygenation was significantly inhibited by treatment with lec-SOD but not by lecithin or rhSOD ($P < 0.01$). Analysis by flow cytometry demonstrated that E-selectin and ICAM-1 were up-regulated by hypoxia/reoxygenation that was inhibited in part by lec-SOD.

Conclusions. The results from this study suggest that incorporation of lec-SOD into organ preservation solutions provides effective protection to endothelial cells against cold ischemia and reperfusion injury following transplantation.

Key words: transplantation, cold organ storage, free radical damage, organ preservation, antioxidants, anastomosis.

Received for publication December 18, 2000
and in revised form February 19, 2001
Accepted for publication March 12, 2001

© 2001 by the International Society of Nephrology

The return of blood flow to ischemic tissue can result in recovery of normal cellular function, but paradoxically, the tissue may become injured during the process of reperfusion. In organ transplantation, prolonged ischemic periods are experienced during cold preservation. Hence, when blood is returned to the ischemic organ following anastomosis, the organ undergoes some level of reperfusion injury. Ischemia/reperfusion injury (IRI) is a nonspecific inflammatory process that is initiated at the endothelial surface of the vasculature and may have an additional impact on post-transplantation events by increasing susceptibility to subsequent acute rejection episodes and the vascular changes associated with chronic rejection [1–3].

A key feature of IRI is the generation of reactive oxygen species resulting from the return of oxygen to ischemic tissue. In vitro evidence has demonstrated that the production of superoxide ($\cdot O_2^-$) on the surface of endothelial cells results from the action of xanthine oxidase, an enzyme that accumulates in ischemic tissue as a consequence of the proteolytic cleavage of xanthine dehydrogenase [4, 5]. Superoxide forms the precursor for other highly reactive free radicals such as hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-), which together may form a potent cytotoxic cocktail capable of inactivating cellular enzymes and initiating inflammatory pathways. The endothelial lining of the organ vasculature plays a critical role in the inflammatory response arising from IRI. In vitro studies have shown that endothelial cells exposed to hypoxia/reoxygenation (to simulate the physiological events of IRI) produce oxygen free radicals resulting in up-regulated expression of leukocyte adhesion molecules, E-selectin, and intercellular adhesion molecule-1 (ICAM-1), thus enabling neutrophil adherence [6–10]. Similarly, these events are observed in vivo where oxygen-free radical production, induced adhesion molecule expression, and neutrophil infiltration have been observed in animal models of organ IRI [11–13]. Further-

more, it is evident from these models of IRI and in clinical renal transplantation that IRI becomes manifest in the microvasculature where the inflammatory changes are most pronounced.

The burst of free radical production at reperfusion overwhelms the cellular capacity for protection against free radical-mediated damage as natural sources of antioxidants (for example, glutathione, ascorbic acid, tryptophane, superoxide dismutase) become depleted during the period of ischemic storage. In transplantation, alleviation of the effects of IRI have been attempted with the development of complex organ preservation solutions such as Euro-Collins, HTK (histidine, tryptophan, α-ketoglutarate), UW (University of Wisconsin), and Marshall's (hypertonic citrate) solution that contain a variety of buffers, slowly permeable solutes to prevent edema, glucose metabolites, and free radical scavengers [14]. Nevertheless, the addition of free radical scavengers to preservation solutions (for example, allopurinol, glutathione, ascorbate) has been used with limited success (because of their short half-lives) and they have not been routinely incorporated into transplantation regimens [15].

Other antioxidant strategies have involved the infusion of superoxide dismutase intravenously to recipients of renal allografts at reperfusion. Early experimental studies demonstrated that superoxide dismutase (SOD) improved early graft function [16, 17], while in clinical renal transplantation, administration of SOD at the time of reperfusion did not appear to benefit renal function in the first week [18], but significantly improved long-term allograft survival [19]. The effectiveness of free radical scavengers could be improved if the agents had a longer pharmacological half-life and if the protection was localized to the endothelial surface where the initial burst of superoxide production is generated following IRI. Indeed, development of such a drug may prove to be of significant therapeutic benefit not only in organ transplantation, but also in other vascular disorders where oxygen free radical-induced damage is of pathological importance.

The aim of this study was to investigate the potential efficacy of a novel agent, lecithinized-recombinant human CuZn-superoxide dismutase (lec-SOD), which has been shown to have a high affinity for cell membranes, to have a longer half-life, and to be a more effective free-radical scavenger than unmodified, recombinant human CuZn-SOD (rhSOD) [20]. The increased affinity of lec-SOD for cell membranes is attributable to the covalent linkage of four molecules of lecithin (a highly cytotropic phospholipid molecule found ubiquitously in cellular membranes [21, 22]) to one molecule of rh-SOD [23]. In renal transplantation, it would be desirable to incorporate lec-SOD into the preservation solution during cold storage and thereby localize its protective capacity to the endothelium. To simulate the physiological conditions experienced in the clinical setting, we have developed a cold hypoxia/reoxygenation model to investigate the effect of lec-SOD on hypoxia/reoxygenation-induced cell death, neutrophil adhesion, and adhesion molecule expression in endothelial cells.

METHODS

Isolation of HUVEC

Umbilical cords were obtained from the Delivery Suite, Department of Obstetrics and Gynaecology (John Radcliffe Hospital, Oxford, UK), and isolation of human umbilical vein endothelial cells (HUVEC) was performed according to the method of Jaffe et al [24]. HUVEC were cultured in M199 medium (Sigma, Poole, Dorset, UK) supplemented with 20% heat-inactivated fetal calf serum (PAA Labs, UK), 2 mmol/L L-glutamine (PAA Labs, Yeovil, UK), 1 × endothelial cell growth factor (Sigma), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA Labs) and incubated at 37°C in an humidified incubator in 95% air and 5% CO_2. Cells were passaged by incubation with phosphate-buffered saline (PBS)/1 mmol/L ethylenediaminetetraacetic acid (EDTA) at 4°C for 10 minutes, followed by a ten-minute incubation at 37°C, and were never used beyond passage 4 in any experiment. Confirmation of endothelial characteristics was determined by the cobblestone morphology of confluent monolayers and by FACS analysis for constitutive surface expression of CD31 and cytokine-inducible E-selectin expression [following incubation with 200 U/mL TNF (Cambridge Biosciences Ltd., Cambridge, UK) for 6 hours].

Culturing the human microvascular endothelial cell line, HMEC-1

A human microvascular endothelial cell line, HMEC-1, was obtained from the Centre for Disease Control (Emory University, Atlanta, GA, USA) [25]. The cells were maintained in culture in MCDB-131 medium (Sigma) supplemented with 15% heat-inactivated fetal calf serum (PAA Labs), 10 ng/mL epidermal growth factor (Sigma), 1 μg/mL hydrocortisone (Sigma), 100 U/mL penicillin, and 100 μg/mL streptomycin (PAA Labs). Cells were passaged at 80% confluence (~days 4 and 5) using the method described for HUVEC.

Source of lec-SOD, rhSOD and lecithin

Lecithinized superoxide dismutase (PSD04), rhSOD, and lecithin were kindly provided by the Seikagaku Corporation (Tokyo, Japan). The pharmacological activity of lec-SOD and rhSOD per 100 μg protein was reported as 300 U and 510 U, respectively (as determined by the xanthine/xanthine oxidase system) [26].

Chemiluminescent analysis of rhSOD and lec-SOD activity

The pharmacological activity of rhSOD and lec-SOD following incorporation into Marshall's organ preserva-

tion solution was determined using a xanthine/xanthine oxidase system based on a method by Skatchkov et al [27]. Two milliliters of Kreb's HEPES buffer was preheated to 37°C and added to a cuvette with 1.0 μmol/L Cypridina luciferin analog (CLA) together with 200 μL of the test sample. Background luminescence levels were measured over a two-minute period in the luminometer (Turner Design TD20-20, Steptech, Stevenage, UK). The generation of superoxide was chemically induced by the addition of 10 μL xanthine oxidase (0.002 U; Sigma) and 80 μL xanthine (400 nmol/L; Sigma), and the level of luminescence produced from superoxide reactivity with CLA was recorded for five minutes. The data were analyzed using AcqKnowledge™ software.

Binding of lec-SOD to endothelial cells

Flow cytometry. Endothelial cells were incubated with lec-SOD and rhSOD incorporated into Marshall's preservation solution (Baxter Healthcare Ltd., Newbury, UK) for 3 and 24 hours at 4°C. Following extensive washing with PBS to remove any unbound lec-SOD or rhSOD, cells were detached (discussed previously in this article) and stained with an anti-human CuZn-SOD monoclonal antibody (mAb; SD-6; Sigma) and detected with a FITC-conjugated goat anti-mouse Ig. All incubations were performed on ice in the dark. Cells were washed twice with PBS and fixed with 1% formalin before data acquisition on a FACSort flow cytometer (Becton-Dickinson, Immunocytometry System, CA, USA) and analysis using CellQuest™ software.

Confocal laser microscopy analysis for localization of bound lec-SOD. A total of 1×10^5 endothelial cells in 200 μL of supplemented medium were spread onto coverslips pre-treated with 50 μg/mL fibronectin solution (Boehringer Mannheim, Lewes, UK) and left overnight in a humidified 37°C incubator. Once cells had firmly adhered, they were cultured until confluent monolayers were obtained (5 to 6 days).

Analysis to test for the internalization of lec-SOD was performed using the Leucoperm™ kit (Serotec Ltd., Oxford, UK) according to the manufacturer's protocol. Briefly, confluent endothelial monolayers were incubated with 50 μg/mL lec-SOD for three hours. Cells were permeabilized in the presence of an anti-human CuZn-SOD mAb (IgG1; SD-6; Sigma), and lec-SOD was detected with a FITC-conjugated secondary goat anti-mouse IgG1 (Sigma) following incubation for 30 minutes in the dark. The cells were washed twice with PBS, fixed with 1% formalin, mounted with Vectashield™ to preserve fluorescence (Vector Labs, Peterborough, UK) and stored at 4°C in the dark. Cellular staining was analyzed on a Zeiss LSM410 confocal laser microscope. Low levels of native SOD were detected in untreated cells following staining with an anti-SOD mAb. An irrelevant isotype control antibody was used to check for cellular autofluorescence and nonspecific binding of the secondary isotype-specific, fluorescence-labeled antibody. Incubation of fibronectin-coated coverslips with lec-SOD showed there was no binding to fibronectin, and incubation of cells with lec-SOD followed by staining with an isotype control mAb showed no lec-SOD autofluorescence (data not shown).

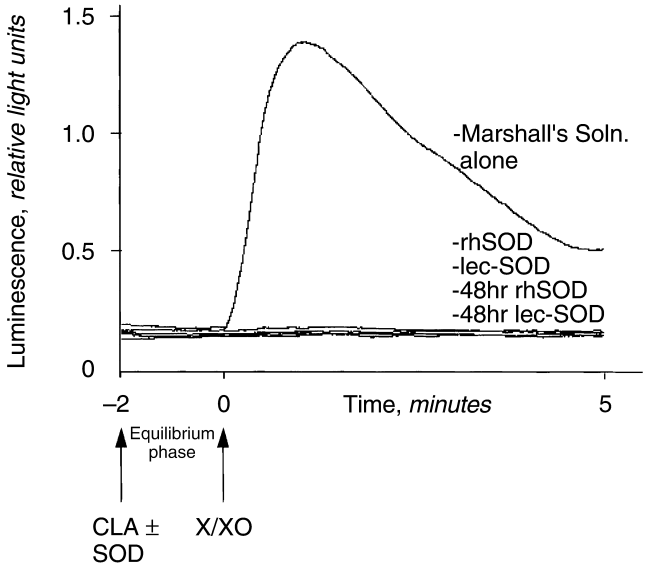

Fig. 1. Effect of recombinant human CuZn superoxide dismutase (rhSOD) and lecithinized superoxide dismutase (lec-SOD) on Cypridina luciferin analog (CLA) chemiluminescence profiles induced by xanthine/xanthine oxidase (X/XO). Background levels of CLA chemiluminescence in the presence of Marshall's solution alone, 30 U/mL rhSOD or lec-SOD were evaluated for two minutes prior to the addition of xanthine/xanthine oxidase (X/XO). Chemiluminescence resulting from X/XO generated superoxide reaction with CLA was assessed for a further five minutes. The addition of 30 U/mL of rhSOD or lec-SOD before and after 48-hour cold storage completely abrogated the superoxide-induced chemiluminescent signal on both occasions the experiments were performed.

Hypoxia/reoxygenation of endothelial cells. Confluent endothelial cultures were placed inside a sealed perspex chamber and hypoxic conditions created by perfusion of the chamber with 95% nitrogen, 5% CO_2 until 0% oxygen was detected by a Class T-7 Teledyne oxygen sensor (Viamed, Keighley, UK) analyzed with a TED 60T oxygen meter (Viamed). Following the period of hypoxia, cells were washed once with PBS to remove unbound lec-SOD and rhSOD, and fresh supplemented medium added and reoxygenation performed at 37°C in a humidified 5% CO_2 incubator to simulate reperfusion conditions. This partially simulates the in vivo conditions in which unbound SOD would be removed from the vasculature of the organ at reperfusion.

Effect of cold hypoxia on endothelial cell viability. Confluent cultures of endothelial cells in six-well plates were incubated under cold hypoxic conditions (95% N_2, 5% CO_2) for 18, 24, and 27 hours in the presence of 1 mL

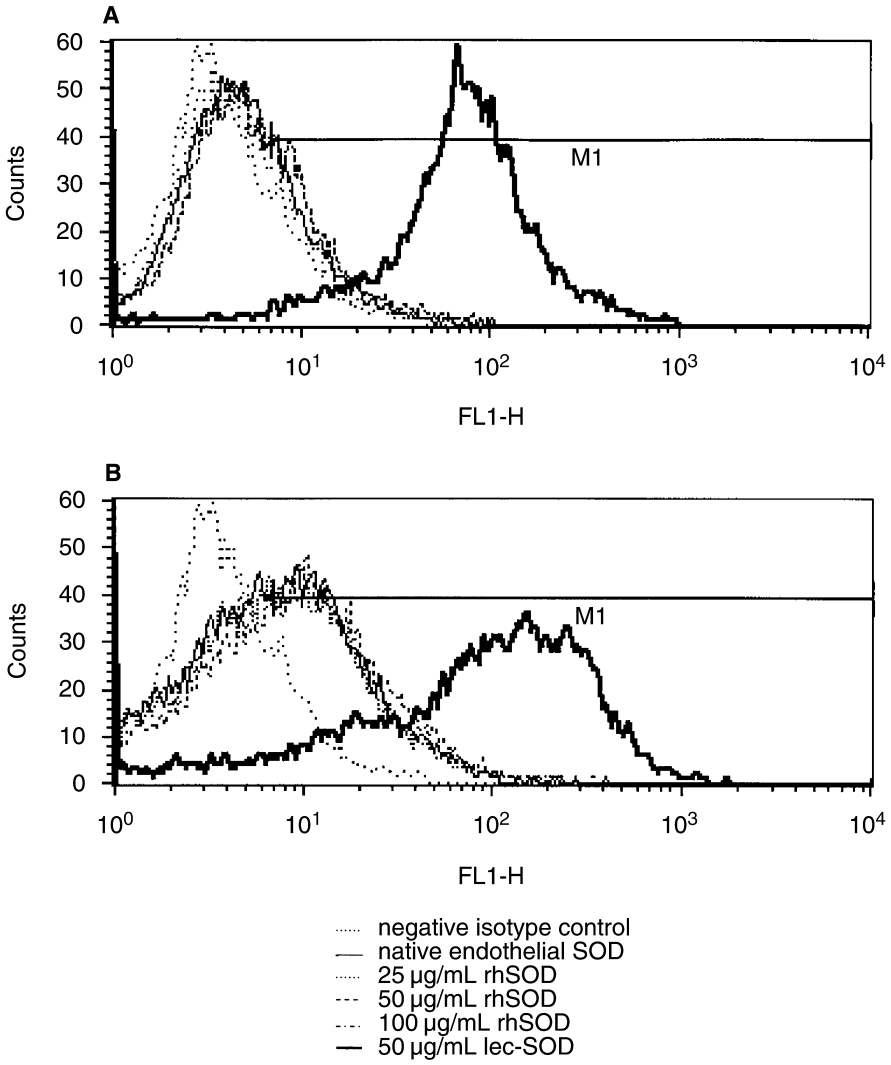

Fig. 2. Lec-SOD binds with higher affinity to HUVEC than rhSOD following incorporation into Marshall's cold preservation solution. HUVEC were incubated with 25, 50, and 100 μg/mL rhSOD at 4°C for (*A*) 3 and (*B*) 24 hours. Exposure of cells to 50 μg/mL lec-SOD was analyzed for comparison. SOD binding was determined by FACS with an anti-SOD mAb detected with a FITC-conjugated goat anti-mouse Ig. Lec-SOD bound with higher affinity to endothelial cells than rhSOD under organ preservation conditions. This experiment was performed in triplicate with similar results obtained on each occasion.

of lec-SOD, rhSOD, lecithin, or lecithin and unconjugated rhSOD in Marshall's solution. Following cold hypoxia, 2 mL of supplemented medium were added to each well for reoxygenation at 37°C in a humidified 5% CO_2 incubator for 24 hours. Following reoxygenation, the remaining adherent cells were carefully detached, and cell viability was determined by Trypan Blue exclusion staining. The experiment was performed in duplicate on three separate occasions.

Purification and labeling of neutrophils. Fifty milliliters of fresh peripheral blood were obtained from healthy volunteers and anticoagulated with 15% EDTA in 4.5 mL Vacutainer™ tubes (Becton Dickinson). Neutrophils were purified using a lipopolysaccharide-free method as previously described [28]. More than 99% viability of neutrophils was observed following Trypan Blue exclusion staining, and neutrophil purity of >95% was obtained as determined by morphological analysis following Rapid Romanowski staining (HD Supplies, UK).

Purified neutrophils were labeled with 50 μCi of sodium chromate (^{51}Cr; Amersham International PLC, Bucks, UK) at 37°C for one hour and were washed and resuspended to a concentration of 1×10^7 cells/mL.

Neutrophil-endothelial cell adhesion assay. Analysis of neutrophil-endothelial cell adhesion (NECA) following cold hypoxia was not possible due to endothelial cell contraction and consequent disruption of a continuous monolayer. Therefore, a method adapted from Ichikawa et al was performed [10]. Briefly, 1×10^4 endothelial cells were seeded onto 96-well plates (Greiner, UK) precoated with 50 μg/mL fibronectin solution (Boehringer Mannheim, Lewes, UK). Upon confluence, endothelial cells were exposed to one-hour of hypoxia (95% N_2, 5% CO_2) and four hours of reoxygenation at 37°C, and then incubated with 5×10^5 neutrophils in a volume of 50 μL for 30 minutes at 37°C after the period of reoxygenation.

Nonadherent neutrophils were removed by three repeated washes and transferred onto a Spot-On filtermat

(Wallac, Milton Keynes, UK), and adherent neutrophils and endothelial cells were lysed with 50 μL 0.1 mol/L sodium hydroxide before transfer to filtermats. Gamma emission was quantified on a Wallac 1205 Betaplate Counter (Wallac), and the percentage of neutrophil adherence was calculated. Each experiment was performed at least three times, with ten samples per experiment.

Adhesion molecule expression following hypoxia/reoxygenation. To determine the adhesion molecules involved in the NECA assay following one hour of hypoxia and four-hours of reoxygenation of HUVEC and HMEC-1, the endothelial cells were stained with antibodies against CD31 (BBA7; British Biotech Ltd., Oxford, UK), P-selectin (G1; [29]), E-selectin (5D11; British Biotech Ltd.) and ICAM-1 (14C11; British Biotech Ltd.), and analyzed by flow cytometry following detection with a fluorescein isothiocyanate-conjugated goat anti-mouse Ig. The cells were fixed in 1% formalin and analyzed by flow cytometry. An irrelevant isotype control antibody (anti-dog Thy-1 [30]) was used as a negative control. Mean fluorescence index was calculated from the percentage of positive cells gated relative to the mean channel fluorescence and analyses were repeated on at least five occasions.

Statistical analyses

Statistical analyses using the Mann-Whitney *U* test were performed to determine whether there were significant differences between untreated and stimulated endothelial cells at a significance level of $P < 0.05$.

RESULTS

Lec-SOD and rhSOD maintain pharmacological activity following storage in Marshall's preservation solution

The pharmacological efficacy of lecithinized superoxide dismutase (lec-SOD) following incorporation into Marshall's organ preservation solution was assessed by a chemiluminescent technique involving the reaction of xanthine/xanthine oxidase (X/XO) with a chemiluminescent probe, CLA..

The addition of Marshall's solution alone had no effect on peak $\cdot O_2^-$ production by X/XO over the five-minute assay period (Fig. 1). In marked contrast, $\cdot O_2^-$-induced chemiluminescence was completely abrogated in the presence of 30 U/mL of either rhSOD or lec-SOD. Furthermore, the capacity of lec-SOD or rhSOD to scavenge $\cdot O_2^-$ was not affected following 48 hours of cold storage in Marshall's preservation solution prior to the assay (Fig. 1). The results demonstrated that incorporation of either rhSOD or lec-SOD into Marshall's preservation solution did not affect their pharmacological potency, even after 48 hours of storage.

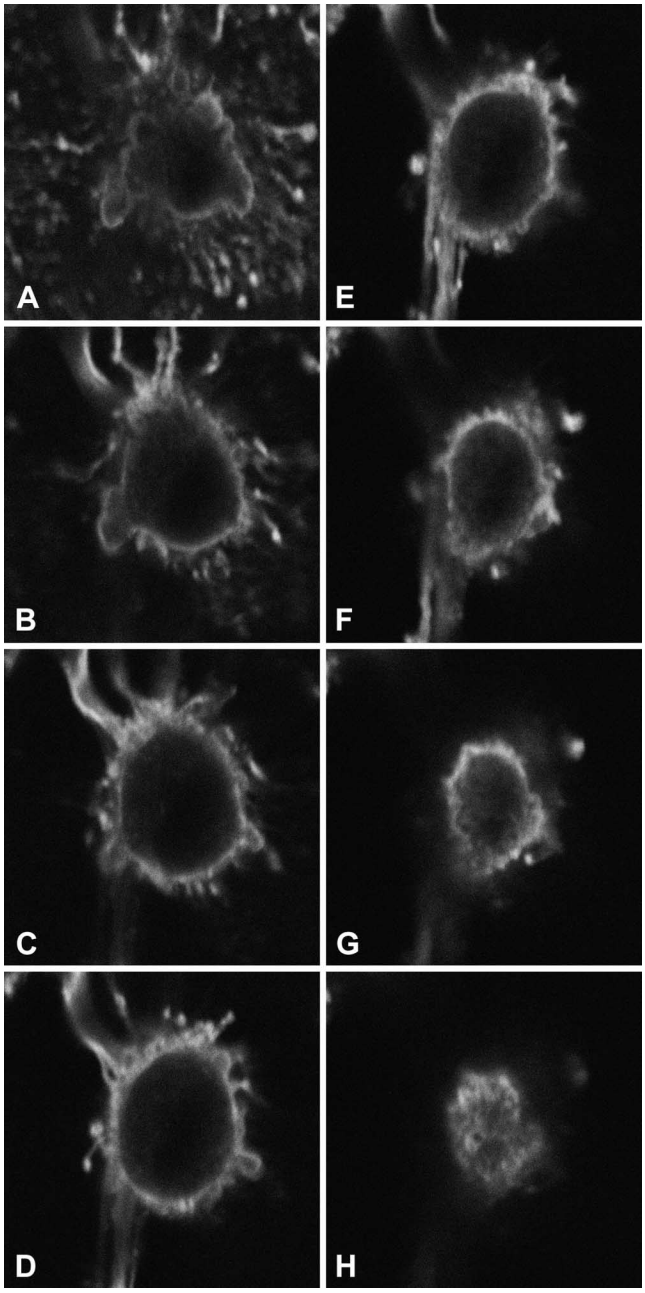

Fig. 3. Serial planar confocal laser microscopy images of lec-SOD binding to endothelial cell surface. HUVEC grown on collagen-coated glass coverslips were incubated with 50 μg/mL of lec-SOD for three hours. Cells were stained with an anti-SOD mAb following permeabilization and staining detected with a FITC-conjugated goat anti-mouse Ig. (*A–H*) Serial laser confocal 3.0 μm sections through a single endothelial cell, demonstrating lec-SOD present on the cell membrane with no detectable SOD inside the cell. This photomicrography series is representative of endothelial cells incubated with lec-SOD for three hours and was performed on at least two separate occasions.

Lec-SOD but not rhSOD binds to endothelial cells in cold Marshall's preservation solution

During the period of organ preservation, an organ may be stored on ice for over 24 hours before transplantation. To recreate similar physiological conditions,

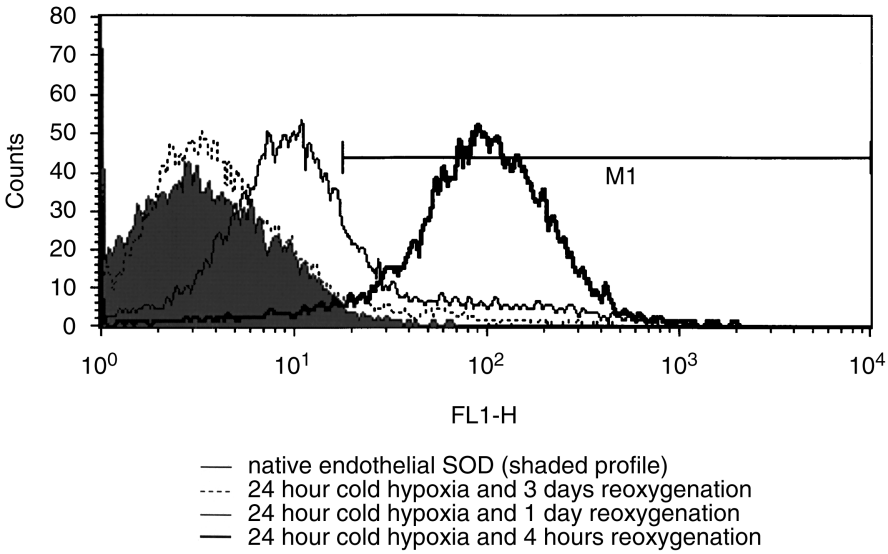

Fig. 4. Analysis of endothelial lec-SOD binding following 24 hours of cold hypoxia and prolonged reoxygenation. HUVEC were incubated with lec-SOD for 24 hours of cold hypoxia then reoxygenated for four hours, one day, and three days to determine the period in which lec-SOD remains bound to the surface. Cells were stained with an anti-SOD mAb and developed with a secondary goat anti-mouse Ig for FACS analysis. The results demonstrated a time-dependent decrease in surface levels of lec-SOD proportional to the period of reoxygenation. This experiment was repeated on three separate occasions, giving similar results.

endothelial cells were incubated at 4°C for 3 and 24 hours with 25, 50, and 100 μg/mL of rhSOD or 50 μg/mL lec-SOD diluted in Marshall's preservation solution. These concentrations of lec-SOD were selected based on studies performed by Igarashi et al, who demonstrated that 100 μg/mL lec-SOD bound with high affinity to endothelial cells after incubation at 37°C for three hours [20].

Incubation of endothelial cells with rhSOD demonstrated no binding above the basal levels of native SOD produced by endothelial cells, whereas high levels were detected by flow cytometry following incubation with 50 μg/mL lec-SOD (Fig. 2). The binding of lec-SOD to the surface of endothelial cells was not affected by shear forces created during removal of endothelial monolayers to form single cell suspensions and the repeated washing steps during antibody staining protocols for flow cytometric analysis. Subsequent titrations of lec-SOD demonstrated that binding to HUVEC and HMEC-1 was detectable at similar levels throughout a range of concentrations (for example, 10 to 100 μg/mL lec-SOD) following 24-hour cold storage (data not shown).

Laser confocal microscopy revealed that following cell permeabilization, lec-SOD was detectable only on the surface of endothelial cells and no intracellular SOD was observed in 3.0 μm serial planar laser images captured through the endothelial cells (Fig. 3)

Effect of hypoxia/reoxygenation on lec-SOD binding to endothelial cells

Human umbilical vein endothelial cells were incubated for 24 hours in conditions of cold hypoxia (95% N_2, 5% CO_2) in the presence of 50 μg/mL lec-SOD and reoxygenated at 37°C for four hours, one day, and three days to determine whether hypoxia/reoxygenation affected surface levels of lec-SOD. In endothelial cells incubated with lec-SOD under cold hypoxic conditions for 24 hours, maximal levels of lec-SOD were detected after four hours of warm reoxygenation, declining in a time-dependent fashion after one and three days of reoxygenation, by which time levels were similar to native SOD levels produced by HUVEC (Fig. 4). Similar results were obtained for binding of lec-SOD to HMEC-1 following hypoxia/ reoxygenation.

Prolonged cold hypoxia and reoxygenation-induced endothelial cell death is partially inhibited by lec-SOD

No significant reduction in cell viability of HUVEC was detected following 24 hours of cold hypoxia and reoxygenation for 24 hours (78 ± 3%) compared with normoxic controls (84 ± 3%; Fig. 5A), but a significant reduction in cell viability was observed following 27 hours of cold hypoxia/reoxygenation (24 ± 4%, $P < 0.01$). However, incubation with 50 μg/mL lec-SOD afforded a significant improvement in cell viability following 27 hours of cold hypoxia (50 ± 7%) compared with HUVEC treated with 50 μg/mL lecithin (21± 3%) and 50 μg/mL lecithin together with rhSOD unconjugated (20 ± 2%, all $P < 0.01$). Similar levels of endothelial cell viability were observed after 30 hours of cold hypoxia/ reoxygenation.

Incubation of HMEC-1 cells for 18 and 24 hours of cold hypoxia and reoxygenation did not result in a significant reduction in cell viability compared with normoxic endothelium (82 ± 7% cells viable). In marked contrast, following 27 hours of cold hypoxia, a significant reduction in cell viability was detected in HMEC-1 cells (27 ± 7%), and this remained significant regardless of treatment ($P < 0.01$). However, in HMEC-1 treated with 50 μg/mL lec-SOD, the reduction in cell viability was less marked (53 ± 7%) and significantly better than treatment with

either 50 μg/mL lecithin (25 ± 6%) or 50 μg/mL lecithin and rhSOD (24 ± 7%, $P < 0.01$; Fig. 5B).

Inhibition of neutrophil adhesion to hypoxia/reoxygenation stimulated endothelial cells following incubation with lec-SOD

Exposure of HUVEC to hypoxia/reoxygenation resulted in a more than twofold increase in NECA (5.6 ± 0.8% to 12.0 ± 2.6%, $P < 0.01$), which was not significantly inhibited with 50 μg/mL rhSOD (10.1 ± 0.9%), 50 μg/mL lecithin (8.4 ± 1.6%), or 50 μg/mL lecithin and rhSOD (8.9 ± 1.9%; Fig. 6A), but treatment with 50 μg/mL lec-SOD significantly abrogated increased NECA (6.3 ± 1.4%, $P < 0.01$; Fig. 6A).

The HMEC-1 cells exposed to hypoxia/reoxygenation exhibited a similar increase in neutrophil adhesion (5.6 ± 1.6% to 12.8 ± 2.7%, $P < 0.01$), which was significantly reduced by treatment of HMEC-1 cells with 50 μg/mL lec-SOD (6.2 ± 1.7%, $P < 0.01$) but not with 50 μg/mL rhSOD (10.7 ± 3.3%), 50 μg/mL lecithin (10.4 ± 3.7%), or 50 μg/mL of unconjugated lecithin and rhSOD (10.6 ± 3.2%; Fig. 6B).

E-selectin and ICAM-1 are involved in NECA following hypoxia/reoxygenation

Fluorescence-activated cell sorter (FACS) analysis was performed to determine endothelial adhesion molecules involved in the increased neutrophil adherence to hypoxia/reoxygenation conditioned endothelial monolayers.

CD31 and ICAM-1 were constitutively expressed on HUVEC, whereas P-selectin and E-selectin were absent (Fig. 7A). Up-regulated expression of ICAM-1 was detected on HUVEC exposed to hypoxia/reoxygenation that was inhibited by 50 μg/mL lec-SOD. E-selectin expression was also induced on HUVEC following hypoxia/reoxygenation, but was only partially inhibited by 50 μg/mL lec-SOD. The induction of P-selectin was not observed.

Constitutive expression of CD31 and ICAM-1 were detected on unstimulated HMEC-1, with no expression of P-selectin or E-selectin (Fig. 7B). Following hypoxia/reoxygenation, a marginal but consistent increase in both ICAM-1 and E-selectin expression was detected on HMEC-1 that was inhibited by 50 μg/mL lec-SOD. Induction of P-selectin was not observed on hypoxia/reoxygenation-conditioned HMEC-1.

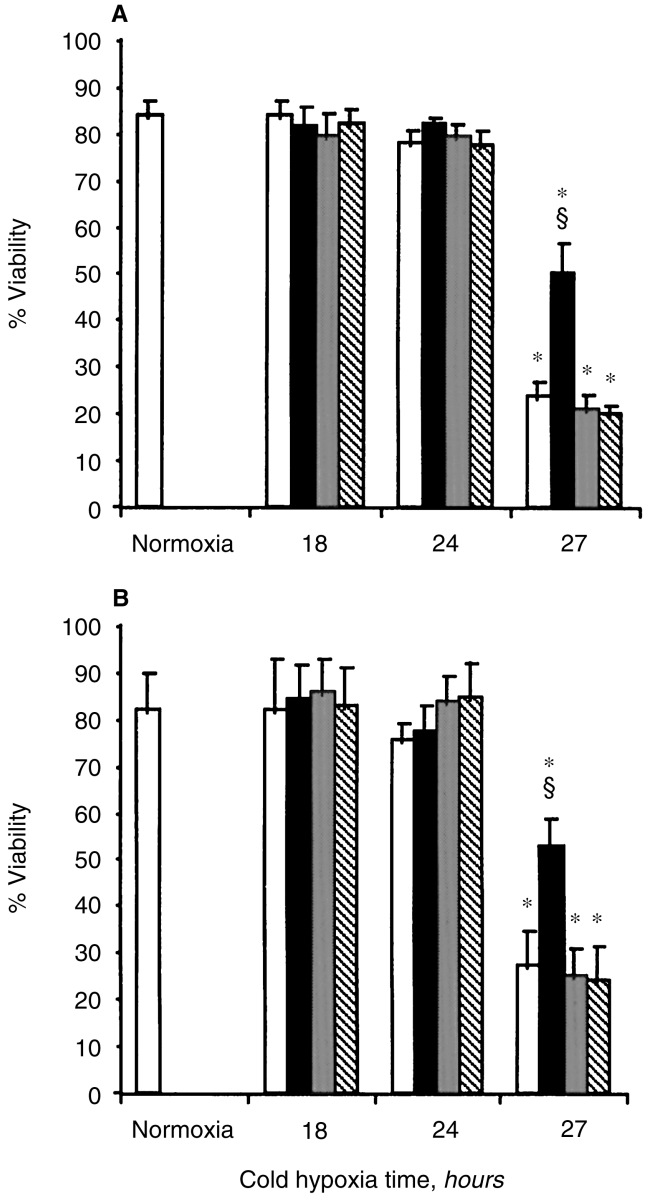

Fig. 5. Viability of HMEC-1 and HUVEC following cold hypoxia and reoxygenation for 24 hours. (*A*) HUVEC and (*B*) HMEC-1 cells were incubated for 18, 24, and 27 hours under cold hypoxic conditions with no (□) or 50 μg/mL of lec-SOD (■), lecithin (▩), or lecithin and rhSOD (▨), and then reoxygenated under normoxic conditions for 24 hours. Cell viability was assessed by Trypan Blue exclusion staining after reoxygenation. Incubation of endothelial cells with 50 μg/mL lec-SOD had a significantly protective effect against 27 hours of cold hypoxia and 24 hours of warm reoxygenation compared with other treatments. Each value represents mean ± SD for three experiments performed in duplicate.

DISCUSSION

The aim of this study was to simulate the events that occur during ischemia/reperfusion injury in renal transplantation using an in vitro endothelial cell model to examine whether lec-SOD would be beneficial in preventing oxygen free radical-derived damage. It was our hypothesis that following flushing through the vasculature of the organ with preservation solution, lec-SOD may be directly targeted to the endothelium during the period of cold storage and exert its protective effect on reperfusion.

We have determined that lec-SOD can be incorporated effectively in organ preservation solution, and can be detected bound to endothelial cells after 24 hours of

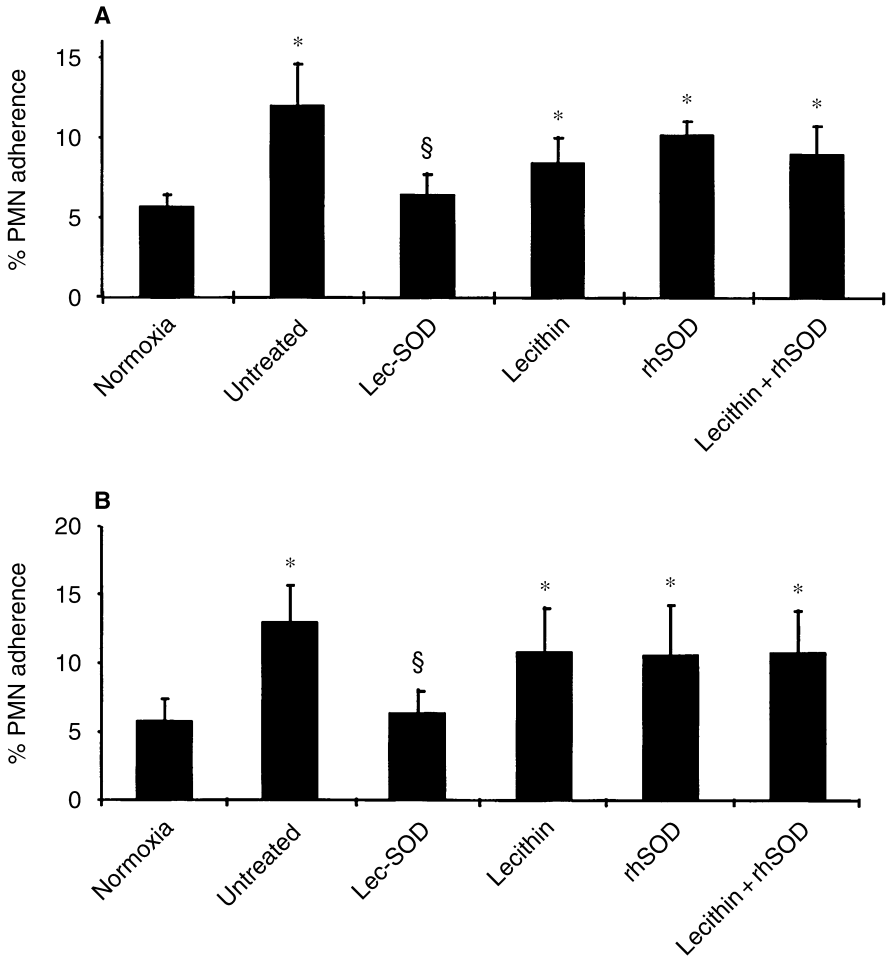

Fig. 6. Comparison of effects of lec-SOD, rhSOD, and lecithin on neutrophil adhesion to HMEC-1 and HUVEC following one hour of hypoxia and four hours of reoxygenation. Confluent monolayers of (*A*) HUVEC and (*B*) HMEC-1 were incubated for one hour under hypoxic conditions either untreated or with 50 μg/mL of lec-SOD, rhSOD, lecithin alone or lecithin and rhSOD, together but unconjugated, and then reoxygenated for four hours. ^{51}Cr-labeled neutrophils were incubated with HUVEC and HMEC-1 after this period for 30 minutes before determining percentage neutrophil adherence. Treatment of endothelial cells with lec-SOD significantly inhibited neutrophil adhesion to both HUVEC and HMEC-1 stimulated by hypoxia/reoxygenation (H/R). Each value represents mean ± SD for 10 measurements, replicated in three experiments. *$P < 0.01$ vs. normoxia; §$P < 0.01$ vs. H/R HUVEC untreated.

cold hypoxic storage. In contrast, rhSOD did not bind to endothelial cells during the 24-hour cold hypoxic period. Once the preservation solution containing rhSOD was removed, washed, and replaced with fresh medium, no protective effects after reoxygenation were observed. This partially simulates in vivo conditions where unbound SOD would be removed from the vasculature of the organ at reperfusion. Lec-SOD was not detectable intracellularly, as demonstrated by confocal laser microscopy and planar laser imaging. However, intracellular uptake of lec-SOD may not be critical, as increased surface levels may be more effective in reducing the insults from ischemia/reperfusion. Exogenous SOD administered immediately prior to reperfusion has been shown to reduce significantly the impact of oxygen free radical-derived damage at the endothelial surface in renal transplantation [16, 17, 19].

Furthermore, after hypoxia and warm reoxygenation, lec-SOD remained on the cell surface and was detectable at maximal levels four hours after reoxygenation, declining in a time-dependent manner after three days. Native levels of CuZn-SOD have been shown to be minimal in the initial period following transplantation of cadaver renal allografts, but a delayed increase in CuZn-SOD production, peaking at day 2 post-transplant, was detected by daily fine-needle aspirate biopsy analysis [31]. These results suggest that grafts would be unable to respond to $\cdot O_2^-$-mediated damage in the critical period immediately following reperfusion. Our results suggest that storage of organs with lec-SOD may provide protection in the initial phase of reperfusion injury when the levels of native SOD within the graft are low. Despite the decline in lec-SOD after three days of reoxygenation in our in vitro model, native levels of SOD produced in a graft may increase sufficiently to provide protection from further damage.

Two clinical trials have been performed with superoxide dismutase in renal transplantation. Pollak et al administered SOD intravenously before and one-hour post-reperfusion, but no beneficial effects were observed with respect to early graft function [18]. In a separate trial, Land et al administered a single, higher dose of SOD intravenously immediately prior to reperfusion. The results demonstrated a significant reduction in first acute rejection

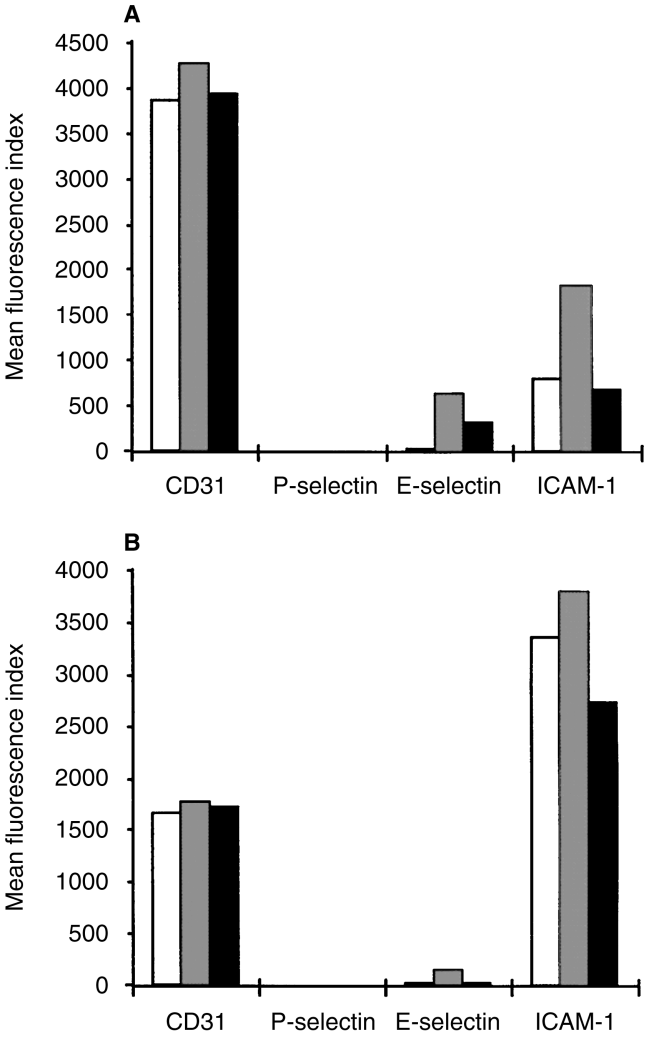

Fig. 7. Phenotypic analysis of HUVEC and HMEC-1 following hypoxia/reoxygenation. Confluent monolayers of (*A*) HUVEC and (*B*) HMEC-1 were incubated for one hour of hypoxia and four hours of reoxygenation either untreated or in the presence of 50 μg/mL lec-SOD. Endothelial cells were stained with mAb against CD31, P-selectin, E-selectin and ICAM-1 and surface antigen expression detected with a secondary FITC-conjugated goat anti-mouse Ig for FACS analysis. The mean fluorescence index was determined from the mean fluorescence intensity and percentage of positive gated cells. Incubation of endothelial cells with lec-SOD appeared to significantly inhibit up-regulation of ICAM-1 and E-selectin following hypoxia/reoxygenation. These observations were representative of at least five separate experiments performed in duplicate. Symbols are: (□) unstimulated; (▩) hypoxia/reoxygenation; (■) lec-SOD.

episodes and improved one- and four-year graft survival compared with placebo controls [19]. Although the inflammatory events associated with ischemia/reperfusion injury may take place within a short time frame, there is accumulating evidence to suggest that these initial events contribute to chronic allograft dysfunction. Many studies have demonstrated that delayed graft function (DGF) resulting from prolonged cold ischemia may significantly diminish long-term renal allograft survival [32–35].

To determine whether lec-SOD had a protective role in hypoxia-induced endothelial cell death, endothelial cells were exposed to 18, 24, and 27 hours of cold hypoxia in Marshall's solution and reoxygenated for 24 hours before assessment of cell viability. Our results demonstrated that following 27 hours of cold hypoxia and reoxygenation, a significant reduction in endothelial viability with increased cell detachment was observed, which was prevented by incubation with 50 μg/mL lec-SOD but not the addition of rhSOD, lecithin, or lecithin and rhSOD in combination. The protective effects of lec-SOD, but not rhSOD or lecithin, suggest that lec-SOD may be effective in preventing cold hypoxia/reoxygenation-induced cell death as a result of oxygen free radical production.

The effects of ischemia/reperfusion injury have been simulated in many in vitro studies by exposing endothelial cells to various periods of hypoxia/reoxygenation. However, direct evidence for the role of oxygen free radicals was provided from electron paramagnetic resonance spectroscopy studies that demonstrated that in human aortic endothelial cells subjected to a period of hypoxia and reoxygenation, superoxide and hydroxyl radicals were detected [4, 5]. These studies demonstrated that the enzyme xanthine oxidase was the primary source of free radical production.

An in vitro model of hypoxia/reoxygenation was adapted from the study of Ichikawa et al to determine the potential efficacy of lec-SOD in preventing neutrophil-mediated damage. Incubation of endothelium with 50 μg/mL lec-SOD during the one-hour hypoxic period significantly attenuated neutrophil-endothelial cell adhesion (NECA) to similar levels obtained for normoxic endothelium, whereas treatment with lecithin alone, rhSOD or lecithin and rhSOD unconjugated did not reduce NECA. Following hypoxia/reoxygenation, up-regulated expression of E-selectin and ICAM-1 were detected by FACS analysis, whereas expression of CD31 and P-selectin was unchanged. Incubation of HUVEC with 50 μg/mL lec-SOD prevented ICAM-1 up-regulation and partially inhibited E-selectin induction. It is likely that inhibition of neutrophil adhesion by preservation with lec-SOD results from the inhibited up-regulation of ICAM-1 and E-selectin expression on hypoxia/reoxygenation-stimulated endothelium. These results support previous studies that demonstrated the participation of ICAM-1 and E-selectin in NECA to hypoxia stimulated endothelial cells using blocking antibody treatment [10, 36].

Following hypoxic treatment of endothelial cells, increased expression of adhesion molecules such as ICAM-1 and E-selectin may be up-regulated by oxygen-free radicals via activation of nuclear factor-κB (NF-κB)–mediated transcription [37]. In addition to hypoxic stimulation, in vitro studies have been performed on endothelial cells exposed to exogenous oxygen free radicals (for example, hydrogen peroxide, X/XO), which have resulted in ele-

vated expression of ICAM-1 and P-selectin and increased binding of neutrophils [38–41]. P-selectin expression has been demonstrated in hypoxia/reoxygenation of endothelial cells [9, 10, 42, 43], but in the current study, P-selectin expression was not detected in HUVEC four-hours post-reoxygenation nor at the earlier time points examined. The inability to detect P-selectin may reflect its transient nature of expression.

Nevertheless, the recruitment and activation of neutrophils by endothelial cells following ischemia/reperfusion may further aggravate inflammatory processes. Upregulated endothelial selectin expression may preferentially recruit neutrophils, which express high levels of sialyl-Lewis x and the high-affinity ligand PSGL-1 on their surface [44–47]. Activation of neutrophils in the microvasculature via molecules such as interleukin-8 and platelet-activating factor result in generation of oxygen free radicals and granular release of proteolytic enzymes [48]. Furthermore, the expression of selectins following ischemia/reperfusion may initiate more severe inflammatory responses, with the selective recruitment of T helper-1 (Th1) cells into the inflamed area [49].

In summary, our results demonstrate that lec-SOD can be incorporated into Marshall's preservation solution and binds with high affinity to endothelial cells. Lec-SOD appears to be effective in protecting endothelial cells against cold hypoxia-induced cell death and upregulated adhesion molecule expression following hypoxia/reoxygenation, and may thus inhibit neutrophil adhesion. This in vitro study supports animal and clinical data demonstrating that the effects of ischemia/reperfusion injury may be attenuated by therapeutic strategies directed against oxygen free radical damage and neutrophil–endothelial cell adhesion molecules in renal transplantation [19, 50–53]. Direct targeting of lec-SOD to the endothelium of the organ prior to transplantation and reperfusion would be a very attractive strategy, but detailed in vivo studies are required before clinical trials could be commenced.

ACKNOWLEDGMENTS

This work was supported by funding from the National Kidney Research Fund (Peterborough, UK) and the Seikagaku Corporation (Tokyo, Japan). The results from this study were accepted for oral presentation and presented at the 2000 XVIII International Congress of the Transplantation Society (Rome, Italy). We are very grateful to Dr. John Forsdyke and Dr. Chris Hawes for their expert support in confocal laser microscopy analysis.

Reprint requests to Dr. Susan V. Fuggle, Nuffield Department of Surgery, University of Oxford, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, England, United Kingdom.
Email: susan.fuggle@nds.ox.ac.uk

APPENDIX

Abbreviations used in this study are: CLA, Cypridina luciferin analog; EDTA, ethylenediaminetetraacetic acid; HMEC-1, human microvascular endothelial cell-1; H_2O_2, hydrogen peroxide; HTK, histidine, tryptophan, α-ketoglutarate; HUVEC, human umbilical vein endothelial; ICAM-1, intercellular adhesion molecule-1; IRI, ischemia/reperfusion injury; lec-SOD, lecithinized superoxide dismutase; NECA, neutrophil endothelial cell adhesion; $\cdot O_2^-$, superoxide; OH^- hydroxyl radical; rhSOD, recombinant human CuZn superoxide dismutase; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; UW, University of Wisconsin; X/XO, xanthanine/xanthine oxidase.

REFERENCES

1. Koo DDH, Fuggle SV: Impact of ischemia/reperfusion injury and early inflammatory responses in kidney transplantation. *Transplant Rev* 14:210–224, 2000
2. Land W: Reactive oxygen species in chronic allograft dysfunction. *Curr Opin Organ Transplant* 14:16–22, 1999
3. Grisham MB, Granger DN, Lefer DJ: Modulation of leukocyte-endothelial interactions by reactive metabolites of oxygen and nitrogen: Relevance to ischemic heart disease. *Free Radic Biol Med* 25:404–433, 1998
4. Zweier JL, Broderick R, Kuppusamy P, *et al*: Determination of the mechanism of free radical generation in human aortic endothelial cells exposed to anoxia and reoxygenation. *J Biol Chem* 269: 24156–24162, 1994
5. Zweier JL, Kuppusamy P, Thompson-Gorman S, *et al*: Measurement and characterization of free radical generation in reoxygenated human endothelial cells. *Am J Physiol* 266:C700–C708, 1994
6. Arnould T, Michiels C, Remacle J: Increased PMN adherence on endothelial cells after hypoxia: Involvement of PAF, CD18/CD11b and ICAM-1. *Am J Physiol* 264:C1102–C1110, 1993
7. Arnould T, Michiels C, Remacle J: Hypoxic human umbilical vein endothelial cells induce activation of adherent polymorphonuclear leukocytes. *Blood* 83:3705–3716, 1994
8. Arnould T, Michiels C, Janssens D, *et al*: Hypoxia induced PMN adherence to umbilical vein endothelium. *Cardiovasc Res* 30:1009–1016, 1995
9. Rainger GE, Fisher A, Shearman C, Nash GB: Adhesion of flowing neutrophils to cultured endothelial cells after hypoxia and reoxygenation in vitro. *Am J Physiol* 269:H1398–H1406, 1995
10. Ichikawa H, Flores S, Kvietys PR, *et al*: Molecular mechanisms of anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. *Circ Res* 81:922–931, 1997
11. Korthuis RJ, Granger DN: Reactive oxygen metabolites, neutrophils, and the pathogenesis of ischemic-tissue/reperfusion. *Clin Cardiol* 16:I19–I26, 1993
12. Grace PA: Ischaemia-reperfusion injury. *Br J Surg* 81:637–647, 1994
13. Weight S, Bell P, Nicholson M: Renal ischaemia-reperfusion injury. *Br J Surg* 83:162–170, 1996
14. Marshall VC, Jablonski P, Scott DF: Renal preservation, in *Kidney Transplantation: Principles and Practice* (4th ed), edited by Morris PJ, Philadelphia, Saunders, 1994, pp 86–107
15. Toledo-Pereyra LH, Simmons RL, Olson LC, Najarian JS: Clinical effect of allopurinol on preserved kidneys: A randomized double-blind study. *Ann Surg* 185:128–131, 1977
16. Koyama I, Bulkley GB, Williams GM, Im MJ: The role of oxygen free radicals in mediating the reperfusion injury of cold-preserved ischaemic kidneys. *Transplantation* 40:590–595, 1985
17. Hoshino T, Maley WR, Bulkley GB, Williams GM: Ablation of free radical mediated reperfusion injury for the salvage of kidneys taken from non-heart-beating donors. *Transplantation* 45:284–289, 1988
18. Pollak R, Andrisevic JH, Maddux MS, *et al*: A randomized double-blind trial of the use of human recombinant superoxide dismutase in renal transplantation. *Transplantation* 55:57–60, 1993
19. Land W, Schneeberger H, Schleibner S, *et al*: The beneficial effect of human recombinant superoxide dismutase on acute and chronic rejection events in recipients of cadaveric renal transplants. *Transplantation* 57:211–217, 1994
20. Igarashi R, Hoshino J, Ochiai A, *et al*: Lecithinized superoxide dismutase enhances its pharmacologic potency by increasing its cell membrane affinity. *J Pharmacol Exp Ther* 271:1672–1677, 1994
21. Mizushima Y, Igarashi R, Hoshi K, *et al*: Marked enhancement in

anti-thrombotic activity of ioscarbacylin following its incorporation into lipid microspheres. *Prostaglandins* 33:161–168, 1987
22. Mizushima Y: Lipo-prostaglandin preparations. *Prostaglandins Leukot Essent Fatty Acids* 42:1–6, 1991
23. Igarashi R, Hoshino J, Takenaga M, *et al*: Lecithinization of superoxide dismutase potentiates its protective effect against Forssman antiserum-induced elevation in guinea pig airway resistance. *J Pharmacol Exp Ther* 262:1214–1219, 1992
24. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins: Identification by morphologic and immunologic criteria. *J Clin Invest* 52:2745–2756, 1973
25. Ades EW, Candal FJ, Swerlick RA, *et al*: HMEC-1: Establishment of an immortalised human microvascular endothelial cell line. *J Invest Dermatol* 99:683–690, 1992
26. McCord JM, Fridovich I: Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* 244:6049–6055, 1969
27. Skatchkov MP, Sperling D, Hink U, *et al*: Quantification of superoxide radical formation in intact vascular tissue using a Cypridina luciferin analog as an alternative to lucigenin. *Biochem Biophys Res Commun* 248:382–386, 1998
28. Haslett C, Guthrie LA, Kopaniak MM, *et al*: Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am J Pathol* 119:101–110, 1985
29. McEver RP, Martin MN: A monoclonal antibody to a membrane glycoprotein binds only to activated platelets. *J Biol Chem* 259: 9799–9804, 1984
30. McKenzie JL, Fabre JW: Studies with a monoclonal antibody on the distribution of Thy-1 in the lymphoid and extracellular connective tissues of the dog. *Transplantation* 31:275–282, 1981
31. Hughes DA, McLean A, Roake JA, *et al*: Free oxygen species (FOS), FOS-scavenging enzyme, P-selectin and monocyte activity in cell populations aspirated from early human renal allografts. *Transplant Proc* 27:2879, 1995
32. Peters TG, Shaver TR, Ames JE, *et al*: Cold ischemia and outcome in 17,937 cadaveric kidney transplants. *Transplantation* 59:191–196, 1995
33. Chertow GM, Milford EL, Mackenzie HS, Brenner BM: Antigen-independent determinants of cadaveric kidney transplant failure. *JAMA* 276:1732–1736, 1996
34. Ojo AO, Wolfe RA, Held PJ, *et al*: Delayed graft function: Risk factors and implications for renal allograft survival. *Transplantation* 63:968–974, 1997
35. Shoskes DA, Cecka JM: Deleterious effects of delayed graft function in cadaveric renal transplant recipients independent of acute rejection. *Transplantation* 66:1697–1701, 1998
36. Paully O, Morliere L, Gris J, *et al*: Hypoxia/reoxygenation stimulates endothelium to promote neutrophil adhesion. *Free Radic Biol Med* 13:21–30, 1992
37. Ghosh S, May MJ, Kopp EB: NF-κB and Rel proteins: Evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 16:225–260, 1998
38. Lewis MS, Whatley RE, Cain P, *et al*: Hydrogen peroxide stimulates the synthesis of platelet-activating factor by endothelium and induces endothelial cell-dependent neutrophil adhesion. *J Clin Invest* 82:2045–2055, 1988
39. Sellak H, Franzini E, Hakim J, Pasquier C: Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. *Blood* 83:2669–2677, 1994
40. Patel KD, Zimmerman GA, Prescott SM, *et al*: Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. *J Cell Biol* 112:749–759, 1991
41. Lakshminarayanan V, Beno DW, Costa RH, Roebuck KA: Differential regulation of interleukin-8 and intercellular adhesion molecule-1 by hydrogen peroxide and tumor necrosis factor-alpha in endothelial and epithelial cells. *J Biol Chem* 272:32910–32918, 1997
42. Closse C, Seigneur M, Renard M, *et al*: Influence of hypoxia and hypoxia-reoxygenation on endothelial P-selectin expression. *Haemostasis* 26:177–181, 1996
43. Pinsky DJ, Naka Y, Liao H, *et al*: Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies: A mechanism for rapid neutrophil recruitment after cardiac preservation. *J Clin Invest* 97:493–500, 1996
44. Lasky L: Selectin-carbohydrate interactions and the initiation of the inflammatory response. *Annu Rev Biochem* 64:113–139, 1995
45. Varki A: Selectin ligands. *Proc Natl Acad Sci USA* 91:7390–7397, 1994
46. Kansas GS: Selectins and their ligands: Current concepts and controversies. *Blood* 88:3259–3287, 1996
47. Varki A: Selectin ligands: Will the real ones please stand up? *J Clin Invest* 99:158–162, 1997
48. Weiss SJ: Tissue destruction by neutrophils. *N Engl J Med* 320:365–376, 1989
49. Austrup F, Vestweber D, Borges E, *et al*: P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. *Nature* 385:81–83, 1997
50. Haug CE, Colvin RB, Delmonico FL, *et al*: A phase I trial of immunosuppression with anti-ICAM-1 (CD54) mAb in renal allograft recipients. *Transplantation* 55:766–773, 1993
51. Hourmant M, Bedrossian J, Durand D, *et al*: A randomized multicenter trial comparing leukocyte function-associated antigen-1 monoclonal antibody with rabbit antithymocyte globulin as induction treatment in first kidney transplantation. *Transplantation* 62: 1565–1570, 1996
52. Negita M, Hayashi S, Yokoyama I, *et al*: Human superoxide dismutase cDNA transfection and its in vitro effect on cold preservation. *Biochem Biophys Res Commun* 218:653–657, 1996
53. Dragun D, Tullius SG, Park JK, *et al*: ICAM-1 antisense oligodesoxynucleotides prevent reperfusion injury and enhance immediate graft function in renal transplantation. *Kidney Int* 54:590–602, 1998